United States Environmental Protection Agency

LABORATORY ANIMAL PROJECT REVIEW

Please note:

1. All information in this LAPR is considered privileged and confidential by the IACUC and regulatory authorities.
2. Approved LAPRs are subject to release to the public under the Freedom of Information Act (FOIA). Do not include proprietary or classified information in the LAPR.
3. An approved LAPR is valid for three years.

LAPR Information

LAPR Title:	Ex Vivo / In Vitro Male Reproductive Assessments and Biomarker Validation using the Male Rat
LAPR Number:	18-09-001
Principal Investigator	Exemption 6
Author of this Document:	Exemption 6/RTP/USEPA/US
Date Originated:	08/28/2015
LAPR Expiration Date:	09/30/2018
Agenda Date:	09/09/2015
Date Approved:	09/21/2015
Date Closed:	

APPROVALS

APPROVER	NAME	APPROVAL DATE	COMMENTS	
	Exemption 6/RTP/USEPA/US by Exemption 6 RTP/USEPA/US	09/21/2015	DMR	
	Exemption 6 Exemption 6 Exemption 6/RTP/USEPA/US by Exemption 6 /RTP/USEPA/US	09/21/2015	DMR	

Administrative Information

1. Project Title (no abbreviations, include species):
Ex Vivo / In Vitro Male Reproductive Assessments and Biomarker Validation using the Male Rat

Is this a continuing study with a previously approved LAPR?
Yes
Please provide the previous LAPR# 15-10-003

2. Programatic Information
a. What Program does this LAPR support? Please provide the Research Program, Project, Task Number and Title.
CSS 12.01 Adverse Outcome Pathway Discovery and Development (AOPDD)
Task 1.3B Development of AOPs for Reproduction in Vertebrates
b. What is the Quality Assurance Project Plan (QAPP) covering this project?
IRP-NHEERL-RTP/TAD/RTB[redacted]/2015-001-r0

3. EPA Principal Investigator/Responsible Employee:

Principal Investigator	***Phone Number***	***Division***	***Mail Drop***
[redacted]	[redacted] ***Lotus Notes Address*** [redacted]/RTP/USEPA/US	TAD ***Branch*** RTB	MD

4. Alternate Contact:

Alternate Contact	***Phone Number***	***Division***	***Mail Drop***
[redacted]	[redacted] ***Lotus Notes Address*** [redacted]/RTP/USEPA/US	TAD ***Branch*** RTB	MD

SECTION A - Description of Project

1. Explain the study objective(s) in <u>non-technical language</u> such that it is understandable by non-scientific persons. <u>Explain how the benefits from the knowledge gained from this research outweigh the costs to the animals used in this research.</u> If this is a continuing study from a previous LAPR, briefly justify the continuation. Please spell out all acronyms and abbreviations with their initial use.

In our previous versions of this LAPR we identified numerous epididymal and Leydig cell toxicants. Our studies of epididymal toxicity employed a 5 day exposure paradigm and a more senstive fertility measure using in utero insemination. This lead us to our first biomarker of effect, the sperm protein SP22. Our work with Leydig cell toxicants involved both in vitro and in vivo assessments. Typically chemicals which decrease testosterone production in vitro are further studied in vivo. These followup studies include both 5 day and 14 day exposures to target compromise in the epididymis and the testis, respectively. More recently we extended our interest in Leydig cell toxicity to the fetal/neonatal testis which may or may not result in persistent latent alterations in the adult. Most recently, we extended our study of Leydig cell toxicity to the aging testis since any chemical which decreases testosterone in a young adult is likely to exacerbate the decline in testosterone production seen with reproductive senescence.

The underlying goal of this research is to identify 'Adverse Outcome Pathways'. These are the molecular events which occur following a specific toxicant exposure and lead to an adverse phenotype (e.g. reduced sperm quantity and quality, decreased fertility). A key point of interest in an adverse outcome pathway is what is now referred to as the 'Molecular Initiating Event'. This is the pivotal alteration that leads to the pathway cascade and ultimately the adverse phenoype. My long term interest has been in identifying biomarkers of effect. The proximate biomarker of effect represents the molecular initiating event. This is the event we seek to identify and validate. Once validated these initiating events (e.g. an unintended rise in fetal testis estradiol) can be used to predict human reproductive risk.

The experimental strategy in this LAPR is to first identify testicular targets in vitro, and then confirm their action in vivo - both during fetal development as well as in the adult. As such we will use our well-known procedure for purifying Leydig cells (the interstitial cells in the testis that produce testosterone) and identify new toxicants which decrease testosterone production. One goal of this is to expand this screen to a 96-well format for high-throughput testing. Toxicants which decrease testosterone significantly and in a concentration-dependent manner will be prioritized for in vivo study. A key feature of the work is the application of proteomics (a quantitative evaluation of individual protein alterations) to elucidate molecular pathways not typically discovered using genomics, receptor-mediated assays, etc. This applies to both in vitro and in vivo studies. Discovering the proteins altered when testosterone is decreased in vitro can provide new candidates for the molecular initiating event with respect to decreased testosterone. The application of proteomics to the in vivo studies should confirm such an event, but also map additional events leading to the adverse phenotype.

2. Scientific rationale for proposed animal use.

a. Why is the use of animals necessary?

For our research, laboratory animals must be utlized albeit to the minimal extent possible. Established cell lines for studying testicular and epididymal effects do not exist because cells lines represent transformed cells which fail to respond with fully differentiated function like normal primary cells and tissue. Validated non-animal tests to predict reproductive toxicity do not exist.

b. Justify the species requested:

We have many years of solid, published data based on our procedures and successful studies in the male rat which initiated with the 1st version of this LAPR in 1988. The reproductive toxicology database has been largely derived from data obtained using rats. Thus, there is no suitable replacement for the rat.

3. How was it determined that this study is not unnecessary duplication?

Google and Pubmed search of terms such as 'sperm biomarker', 'fertility assessed in the rat by in utero insemination', 'proteomics in adverse outcome pathways' generate my publications. Nothing I propose herein has been done before.

<u>SECTION B - In Vivo Procedures</u>

1. Briefly describe the experimental design. Include descriptions of the age, weight and sex of the animals. Supplementary information may be attached at the end of the LAPR, but please include critical information within the body of the LAPR.

This LAPR includes three well-establish experimental designs. Specifics of each design depend on the exposure (both chemical and dose) and the scientific question(s) being addressed. Understand, a positive chemical in Design 1 may or may not be considered for Design 2 and/or Design 3 testing. In fact, only a minimum number of positive chemicals in Design 1 can be considered for In Vivo testing.

Design 1) LEYDIG CELL ISOLATION AND CULTURE. Untreated adult 90-120 day old males are used as donors for Leydig cell cultures. Once testes are removed, the testicular artery is flushed with media to remove blood. The tunica is removed and the parenchyma is is subjected to enzymatic dissociation. The Leydig cells are purified from the dissociated cells using two centrifugation steps (centrifugal elutriation and Percoll density centrifugation).

Design 2) IN VIVO TREATMENT EFFECTS ON SPERM DEVELOPMENT. Experiments involve assessment 5 days after exposure begins to detect effects on post-testicular maturation of sperm in the epididymis, assessment 14 days after exposure begins to detect effects during spermiogenesis in the testis, or a time-course (i.e. 2, 16, and 32 week) assessment following up to 2-week exposure of aging (i.e. 12 month old) animals. Depending on the particular hypothesis being tested for each experiment, males (control and two dose groups) are assessed for mating behavior or fertility by in utero insemination (IUI). Further reproductive assessments include analysis of organ weights, histology, serum hormones, testis/sperm proteomics, and culture of Leydig cells. To determine to what extent alterations observed in vivo are due solely to decreased testosterone, silastic capsules containing testosterone are sometimes implanted to maintain normal circulating levels of testosterone in the animal.

Design 3) PRENATAL DEVELOPMENT. To assess the effect of in utero exposure on prenatal development of the testis and peristent alterations at adulthood. For this, ~70-day-old pregnant dams (control and two dose groups) are exposed from gestation day (GD) 13 through GD 19. On PND 1, testes are collected from male pups for multiple evaluations. Male offspring are also evaluated at puberty (PND56) and adulthood (PND 90).

2. Justify the number of animals. Include explanation (e.g., biological, statistical, regulatory rationale) for the number of animals needed for each treatment group, and the overall number requested for the duration of the LAPR.

Based on years of experience the number of animals used in all studies is the minimum required to attain statistical significance for that procedure, $p<0.05$.

1). Untreated adult males for isolation and culture of Leydig cells (Design 1)
We will use 6 males 10 times a year to obtain enough testis tissue for Leydig cell cultures. The recovery (20 million Leydig cells/6 animals) is enough for one experiment. This in vitro need equates to 60 males per year. For 3 years, 180 needed. Cat. C=180 males.

2) Treated males (young=PND56, adult=PND90 and aging=12 month and beyond) for routine male reproductive assessments including mating behavior, IUI, and histopathology (Designs 2 and 3)
Each year we will evaluate treated males in three studies. Each study will consist of a control and two treatment groups with 8 animals per group. Four of eight animals will be used for mating behavior or IUI. Four of eight animals per group will be perfused for histopathology (Cat.D). Thus, for each year then we need 72 males (8/group x 3groups x 3 studies). For 3 years, 216 needed. Cat.C = 108 males. Cat D = 108 males. Note: eight males will be transferred from the previous LAPR.

3) Adult females for mating behavior evaluations and IUI (Design 2)
For each mating behavior or IUI, we need twice as many females as males as roughly 60% of the females are actually receptive on the day of mating or insemination. That is, for every male evaluated, we need one female to use for mating or undergo IUI (Category D) plus one extra (Category C). As per above, we need 81 males over 3 years; thus, we need 162 females needed for IUI. Cat.C = 81 females, Cat. D= 81 females.

4) Vasectomized teaser males for mating behavior evaluations and IUI (Design 2)

Each year we will vasectomize a new cohort of males to serve as teasers for the females synchronized for IUI as well as those sycnhronized for mating behavior evaluations. For each experiment we conduct 9 evaluations /inseminations at a time, thus we need 9 teaser males and, accordingly, we vasectomize 9 rats at a time. The vasectomized males are sexually active for about 3 months, so we need new animals 3 times a year or 27 males per year. For 3 years, 81 needed. Cat.D=81 males.

5) Timed-pregnant females for prenatal exposures and postnatal day 1 as well as pubertal and adult evaluations (Design 3)
Each year we plan to conduct a single experiment comprising a control and two treatment groups, with a minimum of 8 litters per group (8 litters/group x 3 groups x 2 time points (PND1 and pubertal/adult) = 48 timed-pregnant females/year; 144 timed-pregnant females over 3 years). We estimate 480 PND1 pups per year (48 litters x 10 pups/litter) or 1440 pups over 3 years.

Overall animal needs for each item above are tabulated here.

	TOTAL	CATEGORY
Untreated males (Leydig cells)	180	C
Treated young/adult/aging males	108	C
Treated young/adult/aging males	108	D
Adult females	81	C
Adult females	81	D
Teaser males	81	D
Timed-pregnant females	144	C
PND1 pups	720	C
PND56 / PND90 offspring	720	(included above)
TOTAL	1233	Category C
	270	Category D

3. State how many animals over the study period are expected to be used under the following three categories of pain/distress (USDA nomenclature as defined in the instructions): Please enter numbers only.

Categories	Adults	Offspring
C) Minimal, transient, or no pain/distress:	513	1440
D) Potential pain/distress relieved by appropriate measures:	270	
E) Unrelieved pain/distress:		

4. Does this LAPR include any of the following:

☐ Restraint (>15 Minutes) ☒ Survival surgery
☐ Food and/or water restriction (>6 Hours) ☒ Non-survival surgery

a. Please provide a scientific justification. Describe how animals will be monitored, how health status will be tracked, and what records will be maintained.
We vasectomize to evaluate receptivity of females synchronized with LHRH. IUI is performed to provide a more sensitive measure of male fertility. All animals subject to survival surgery are given post-operative analgesics and monitored daily for one week. Surgical records are maintained in the laboratory.

5. Category C procedures. Describe each procedure separately, include details on the following:
a. Treatments (e.g., dosages, duration of exposure, route, volume, frequency):
Dosing solutions will be made in water, corn oil (Food Grade, 100%), 15% ethanol in water, or 30% DMSO in water. Most chemicals are given by oral gavage at 0.5 ul/g body weight; each male/pregnant female will receive approximately 0.2 ml of dosing solution. Ethane dimethanesulphonate (EDS) is adminstered as a single IP dose in less than 0.4 ml.

Males will be administered test chemical daily by oral gavage for 5 or 14 days with the exception of EDS which is given as a single IP dose (Design 2).

Pregnant females will be dosed with test chemical by oral gavage daily from GD 13 through GD 19 (Design 3).

Test chemicals (administered by gavage)

Prochloraz	maximum dose of 100 mg/kg/day
Bisphenol A	maximum dose of 100 mg/kg/day
Diethyl hexyl phthalate	maximum dose of 100 mg/kg/day
Simvastatin	maximum dose of 100 mg/kg/day
Betamethasone	maximum dose of 100 ug/kg/day
Anilazine	maximum dose of 100 mg/kg/day
Cyanazine	maximum dose of 100 mg/kg/day
Metconazole	maximum dose of 100 mg/kg/day
Fenvalerate	maximum dose of 100 mg/kg/day

Ethane dimethane sulphonate maximum dose of 75 mg/kg (single IP dose only)

Adult females used for mating behavior or IUI (Design 2) will be administered luteinizing hormone releasing hormone (LHRH) agonist in order to synchronize estrous cycles, and provide sexually receptive females for experimentation. LHRH agonist (80 ug sc) will be administered in 0.1 ml Dulbecco's phosphate buffered saline (DPBS) 115 hours prior to evaluation of sexual receptivity or natural breeding.

b. Survival Blood Collections (method, volume, frequency):

N/A

c. Testing methods (including non-stressful dietary restrictions/modifications, mild non-damaging electric shock):

N/A

d. Animal restraint and confinement beyond routine housing and handling. Include a description of the type of restraint device, acclimation to device, duration of restraint:

N/A

e. Breeding for experimental purposes (e.g. length of pairing, number of generations):

In-house breeding will be used to evaluate mating behavior and fertility via natural mating (Study Design 2). Estrous cycles of adult females will be synchronized by administration of 80 ug LHRH agonist in 0.1 ml DPBS via sc injection 115 hours prior to placing in the male's cage. Treated males will be paired with receptive females for one hour to monitor mating behavior afterwhich males will remain with the female overnight. Fertility will be evaluated on GD13. Dams will not be allowed to go to term (i.e., there will be no live births).

f. Describe how animals will be identified and monitored. Include description of identification procedures. (For example, if transponders are used, how are the animals prepared?) Include frequency of observations and by whom:

Animals are identified using ear-tags. Animals that undergo surgery are monitored by laboratory staff (listed in Section E) daily for a week. All dosed animals will be weighed and monitored daily by laboratory staff (Section E) to ensure there are no ill effects from treatments.

6. Non-surgical Category D or E procedures. Describe each procedure separately, include details on the following (Also fill in Section B.9).

a. Treatments (e.g. dosages, duration of exposure, route, volume, frequency):

N/A

b. Blood Collection (Provide a description of the procedure including method, volume, and frequency if appropriate. Indicate if the procedure is survival or terminal. Include preparatory methods, descriptions of incisions, etc.):

N/A

c. Testing methods:

N/A

d. Restrictions placed on the animals' basic needs (e.g., food and/or water restriction, light cycles, temperature). Provide details regarding the length of restriction. Describe the method(s) for assessing the health and well-being of the animals during restriction. (Amount of food or fluid earned during testing and amount freely given must be recorded and assessed to assure proper nutrition.):

N/A

e. Describe how animals will be monitored (e.g., frequency of observations, by whom):

N/A

f. Analgesia (Category D Procedures) - list drugs, dosages, route of administration and frequency:

N/A

g. If treatment-related deaths are expected, this must be thoroughly justified. Death as an endpoint is highly discouraged:

N/A

7. Surgical Category D and E procedures. Indicate if the surgery is survival or terminal. Describe each surgical procedure separately, include details on the following (Also fill in Section B.9)

a. Complete description of surgical procedure including presurgical preparation, aseptic technique, surgical closure, etc:

All surgeries are performed under a fume hood; sterile gloves are worn and sterile surgical drapes are used. Surgical instruments are disinfected (Synphenol-3) and autoclaved packs of surgical instruments are prepared prior to surgery date. Each pack is used for no more than 5 animals. Glass bead sterilization is done between animals. All surgical sites are prepared with clippers and rinsed with 70% ethanol followed by a betadine prep 3 times prior to incision. Thermal support is provided using a re-circulating water blanket.

Vasectomy.

Males are vasectomized to serve as teasers for synchronized females. A mid-abdominal incision is made, and the vas deferens is exposed. Warm, sterile saline is used to rinse the tissues prior to closure to prevent adhesions. Absorbable suture material (4-O) is used to ligate the vas. The abdominal musculature is closed with absorbable (3-O) suture material and the skin is closed using surgical staples or wound clips. Suture materials include chromic gut, coated vicryl (polyglactin), polydioxanone, or polyglycolic acid, and are provided in sterile packets. Staples or wound clips will be removed 9 days post-surgery.

In utero insemination (IUI).

This methodology is applied to Design 2. Estrous cycles of adult females will be synchronized for in utero insemination by administration of 80 ug LHRH agonist in 0.1 ml DPBS via sc injection 115 hours prior to evaluation of sexual receptivity. Sperm from control and treated animals (or sperm treated in vitro) are inseminated into the uterine horns of receptive adult females. For this, a mid-ventral incision is made, and the uterine horns are exposed. Warm, sterile saline is used to rinse the tissues prior to closure to prevent adhesions. Sperm sample from a donor male is injected into the uterine horn. A cauterizing tip is used to close the point of injection into the uterine horn. The abdominal musculature is closed with absorbable Chromic gut (3-O) and the skin is closed using surgical staples or wound clips. Females are killed on post-insemination day 9 for evaluation of uterine implants and ovarian corpora lutea. (No neonates are produced.) Females which are not receptive after a second synchronization attempt will be euthanized or transferred to an approved LAPR.

Silastic capsule implantation.

To control for effects of decreased testosterone by certain chemical exposures (e.g. simvastatin), 1.0 cm pharmaceutical grade testosterone-filled silastic capsules will be inserted under the skin in the center of the back and closed with surgical staples. Staples will be removed 10-14 days post-surgery.

Vascular perfusion.

This is a terminal procedure. Once the animal is under a surgical plane of anesthesia (confirmed by multiple toe pinches), the left ventricle of the heart is exposed. An 18 g needle is connected to a hanging bottle of phosphate buffered saline. The valve is opened so that the saline begins to flow and the needle is inserted into the ventricle. The right atrium is cut and the vasculature is perfused with saline. The animal expires during this perfusion. Next, flow from the bottle containing the fixative used to fix the reproductive organs is is turned on and the saline valve is shut off. Once the reproductive organs have fixed adequately the flow is shut off and the needle is removed.

b. Anesthetic regimen (Drugs, dosages, volume, route of administration and delivery schedule). The use of paralytic or neuromuscular blocking agents w/o anesthesia is prohibited:

Isoflurane (5% to induce, 3% to maintain) administered w/ oxygen via inhalation from a vaporizer routed to an inhalation chamber (to induce) / nose cone (to maintain) until surgical plane is achieved. A surgical plane is identified by absence of withdraw reflex.

c. Postoperative care (thermal support, special feeding, responsible personnel, removal of sutures/staples, frequency and duration of monitoring including weekend and holiday care):

Following analgesics, a re-circulating warm water blanket is used to minimizes stress and discomfort upon recovery. The animals are monitored closely by laboratory staff until they are fully recovered and returned to the animal room. We (laboratory staff) examine the status of the animals in the animal room daily the first week and weekly thereafter until end of study. All cages of animals immediately post-operative are marked with blue plastic hang tags (labelled with PI, contact information, and date of surgery) on cage card holders. Tags are removed upon complete healing of incision (5-7 days).

d. Post operative analgesics (drugs, dosage, and volume and route of administration, frequency):

All animals are given analgesics via a combination of 0.05 mg/kg buprenorphine + 5.0 mg/kg carprofen subcutaneously immediately after induction of anesthesia. Animals are dosed with oral carprofen (2 mg tablet) once daily for 2 additional days. If pain/discomfort appears to be inadequately controlled (e.g. stretching) with one tablet/day, a second tablet will be administered. Note: to acclimate the rats to tablets, blank tablets without carprofen will be offered to the rats in the days prior to surgery.

e. Will any animal be subject to more than one surgical procedure over the course of its lifetime, either here at NHEERL or elsewhere?

○ Yes ● No

f. Identify any surgical procedures performed at other institutions or by vendors:

N/A

8. Humane interventions (for treatments/procedures in all categories).

a. What resultant effects, if any, do the investigators expect to see following procedures or treatment? Please include transitory as well as permanent effects. Examples might include lethargy, ataxia, salivation or tremors. Indicate the expected duration of these effects.

Based on considerable previous experience, we do not expect any deleterious effects from the surgeries or chemical exposures described herein. As animals age, we occassionally see an animal with a skin lesion or growth that is reported and removed from study. Pregnant dams at term will be monitored for parturition. Any animal health issues will be referred to the veterinarian.

b. State the criteria for determining temporary or permanent removal of animals from the study. Describe actions to be taken in the event of deleterious effects from procedures or chemical exposures. Describe actions to be taken in the event of clinical health problems not caused by procedures or exposures.

If we determine through consultation with the veterinarian that pain or suffering may ensue, the animal will be euthanized. Criteria include deteriorating body condition, lethargy, shivering, ruffled fur, arched back, unstable movement, and lack of interest in food or water. Dams exhibiting dystocia will be euthanized.

9. Alternatives to pain and distress (Category D and E Procedures only). Provide narrative regarding the sources consulted to ascertain whether acceptable alternatives exist for potentially painful/distressful procedures. Include databases searched or other sources consulted, the date of the search and years covered by the search, and key words and/or search strategy used. Assistance with searches is available through the EPA Library Staff.

By searching PubMed from 2000-present using Keywords "artificial insemination in the rat" and "vasectomy of the male rat" we could find no data whereby a surgery can be performed without causing some degree of postoperative pain. No reliable procedures are available to do vasectomy or artificial inseminations which produce less pain or stress than those used in this study.

SECTION C - Animal requirements

Describe the following animal requirements :

1. Indicate the number of animals required over the study period for this protocol. Please enter numbers only.

a. Animals to be purchased from a Vendor for this study: 775

b. Animals to be transferred from another LAPR: 8

LAPR Number that is the source of this transfer: 15-10-003

c. Animals to be transferred from another source:

d. Offspring produced onsite (used for data collection and/or weaned): 1440

e. TOTAL NUMBER of animals for duration of the LAPR 2223

2. Species (limited to one per LAPR): Rat(s)

3. Strain: Sprague Dawley rat(s), Brown Norway rats

Describe special requirements for animals with altered physiological responses (e.g., genetically altered, aged)

Brown Norway rats are used for our aging studies

4. Sources of animals:

Charles River for Sprague Dawley, National Institute of Aging for Brown Norway

5. Provide room numbers where various procedures will be performed on animals:

Exemption 6
Exemption 6
Exemption 6

6. Will any animals be housed in areas other than the animal facility longer than 12 hours? If so, state location. Such areas require prior IACUC approval as a satellite facility before LAPR can be reviewed.

N/A *Room Numbers:*

7. Describe any transportation and containment methods involved in moving animals between EPA buildings, or between EPA and other institutions (excluding any commercial shipments)

N/A

8. Describe any unusual housing or husbandry requirements, or acclimation requirements. Justify any treatment beginning less than 3 days after arrival.

One room designated for reversed lighting; lights off at 9:00 AM EST

9. Describe special assistance requested of the animal contract staff, including procedures and dosing. NOTE, this request must be submitted separately to the Animal Resources Program Office (ARPO)

N/A

10. Housing and Enrichment.

The IACUC encourages the use of environmental enrichment whenever possible (see IACUC website for details). Provide details on how the animals will be housed, including type of cage (e.g., solid bottom or wire screen), bedding material, number of animals per cage, and environmental enrichment. Note that housing rodents individually without environmental enrichment requires justification.

Animals housed 1 per cage on heat-treated pine shavings with Enviro-Dri. Single housing is recommended to eliminate fighting between males. It is also necessary to evaluate mating behavior in females and following surgery in either males or females.

SECTION D - Agents Administered to Animals

1. Identify all hazardous and non-hazardous agents to be administered to living animals. For agents requiring a Health and Safety Research Protocol (HSRP), provide the title of the approved HSRP for each such agent. If no protocol is required for an agent deemed potentially hazardous (e.g. nanoparticles, recombinant DNA), describe the safety precautions to be used.
Provide maximum dosing levels and route-appropriate LD50s (where available) for each agent used for dosing.

Isoflurane gas mixed with oxygen as anesthetic - approved for use via amendment to Health and Safety Protocol "Biochemical and Histological Evaluation of Male Reproductive Toxicants".

Carprofen, pharmaceutical grade, oral 2 mg s.i.d. or b.i.d. (tablet); 5 mg/kg sc; oral LD50=149 mg/kg (sc LD50 data unavailable)

Buprenorphine, pharmaceutical grade, 0.05 mg/kg sc; oral LD50>1g
(sc LD50 data unavailable)

Testosterone, pharmaceutical grade, 1.0 cm in silastic capsule. oral LD50>5000 mg/kg
(sc LD50 data unavailable)

[des-Gly10, D-Ala6]-LH-RH ethylamide acetate salt hydrate (≥97%, HPLC grade)
(LHRH agonist), 80 ug given in 0.1 ml Dulbecco's Phosphate Buffered Saline via sc injection. No toxicity data but historically, no adverse effects at this dose.

Test chemicals	Maximum dose	Oral LD50
Prochloraz	100 mg/kg/day	2000 mg/kg
Diethyl hexyl phthalate	100 mg/kg/day	30,000 mg/kg
Simvastatin	100 mg/kg/day	5000 mg/kg
Bisphenol A	100 mg/kg/day	2400 mg/kg
Betamethasone	100 ug/kg/day	1180 mg/kg
Anilazine	100 mg/kg/day	3000 mg/kg
Cyanazine	100 mg/kg/day	334 mg/kg
Metconazole	100 mg/kg/day	1459 mg/kg
Fenvalerate	100 mg/kg/day	451 mg/kg

Ethane dimethanesulfonate (CAS 4672-49-5) Maximum dose = 75 mg/kg (ip); LD50 = 150 mg/kg (ip). Administered in 30% dimethylsulfoxide (DMSO, pharmaceutical grade) in sterile water.

No reported adverse effects at these doses.

Vehicles for test chemicals include water, corn oil (Food Grade, used within one year), 15% ethanol in water, or 30% DMSO in water.

Approved safety precautions (gloves, eye protection, laboratory coat) are used during preparation and handling of test chemicals.

2. Describe compounds to be administered to animals.

a. Are all substances pharmaceutical grade? If not, provide a scientific justification for the use of non pharmaceutical grade compounds.
Simvastatin is pharmaceutical grade. The remaining test chemicals are not available as pharmaceutical grade.

b. Describe any plans to administer human or animal tissues, blood or body fluids to the animals in the LAPR. Provide information to assure that such material is pathogen free. Indicate what safety precautions are necessary for handling the material.
N/A

c. Provide a statement regarding any safety precautions necessary for handling any of these materials.

Approved safety precautions are used during the preparation and handling of test chemicals, i.e. gloves, eye protection, and laboratory coat.

NOTE: Any unresolved health/safety questions which arise during IACUC review of this LAPR will require consultation with the Safety, Health, and Environmental Management Office.

SECTION E - Personnel Training and Experience

1. Identify all project personnel conducting animal experimentation. Specify the techniques for which they have responsibility, and their relevant training and experience. Additional personnel may be added to the table below as a group (by Division) for Category C procedures. By so doing you are giving assurance that these personnel have received all required training and are qualified to perform the Category C techniques requested.

Use this area to type in additional personnel information not available in the table drop-down lists:
N/A

Hint: *The names in the first 2 lines of the table below are filled automatically from the Principal Investigator & Alternate Contact fields. A new line will be made available when a name is selected & upon leaving the name field (i.e. tabbing or clicking in another field).*

NAME	ROLE	SPECIFIC RESPONSIBILITY	RELEVANT TRAINING
Exemption 6	Principal Investigator	Experimental Design, Oversight, and In Utero Insemination	35 years experience. All NHEERL-required training completed.
Exemption 6	Technical Staff	Vasectomy, Dosing, Mating evaluation, LHRH synchronization	30 years experience. All NHEERL-required training completed.
Exemption 6	Technical Staff	Vacular Perfusion	30 years experience. All NHEERL-required training completed.
Exemption 6	Post-Doc	Dosing	All NHEERL-required training completed.
Exemption 6	Technical Staff	Dosing, vasectomy and in utero insemination	25 years experience. All NHEERL- required training complete
Toxicity Assessment Division	Tech Support	Category C Procedures	All NHEERL required training is complete.

SECTION F - Animal Breeding Colonies

This section pertains to the breeding of animals for maintenance of ongoing animal colonies. Do not include breeding that is part of experimentation and accountable under Section C.

Describe:

1. Estimated number of breeding pairs and liveborn per year N/A

2. Breeding protocols and recordkeeping N/A

3. Methods for monitoring genetic stability N/A

4. Disposition of all offspring and retired breeders that are not used in accordance N/A

with the procedures described in this LAPR

SECTION G - Euthanasia

1. When will the animals be euthanized relative to experimental procedures?

Animals are euthanized shortly after the experimental phase is complete.

1) Anesthesia plus cervical dislocation -
Males used for all in vivo studies and males used for in vitro Leydig cell culture are euthanized when tissues (testes, epididymides) have been recovered (i.e., non-survival surgery).
Females that have undergone IUI are euthanized on GD 9 (i.e., 9 days post-insemination).
Females used for mating evaluation and fertility by natural mating are euthanized on GD 13.
Time-pregnant females treated during gestation are euthanized on PND1
Females which are unsuccessful after a second synchronization attempt

2) Anesthesia plus perfusion-
Males used for histological evaluations in vivo studies

3) Decapitation
Pups born on PND1

2. Describe the euthanasia techniques:

Method(s): Anesthesia plus cervical dislocation, Anesthesia plus perfusion (animals used for histology only), Decapitation (PND1 pups only; backup scissors will be available)

Agent(s): Isoflurane
Dose (mg/kg): 5% mixed with oxygen until breathing ceases
Volume: As needed
Route: Inhalation

Source(s) of information used to select the above agents/methods:

2013 AVMA Guidelines on Euthanasia.

3. Provide justification and references for any euthanasia agent or method that is not consistent with recommendations of the American Veterinary Medical Association (AVMA) Guidelines for Euthanasia (e.g., cervical dislocation or decapitation without anesthesia; cervical dislocation in rodents weighing more than 200 grams).

N/A

4. Describe how death is to be confirmed.

Prolonged absence of breathing

SECTION H - Disposition of Used and Unused Animals

Describe the disposition of any animals remaining after project completion.

Euthanized by Animal Care Contractor

The IACUC encourages investigators to reduce the overall number of animals used at NHEERL. Would you consider transferring any unused animals from this LAPR to another approved LAPR?

○ Yes ● No

SECTION I - Assurances

1. Animals will not be used in any manner beyond that described in this application without first obtaining formal approval of the IACUC.
2. All individuals involved in this project have access to this application, are aware of all EPA policies on animal care and use, and are appropriately trained and qualified to perform the techniques described.
3. Thorough consideration of the three "R"'s (Replacement, Reduction, Refinement) has been given, as applicable, to a. the use of animals, and b. procedures causing pain or distress (with or without analgesia/anesthesia), including death as an endpoint. The minimum number of animals required to obtain valid experimental results will be used.
4. The Attending Veterinarian has been consulted in regard to any planned experimentation involving pain or distress to animals.
5. The IACUC and Attending Veterinarian will be promptly notified of any unexpected study results that impact the animals' well-being, including morbidity, mortality and any occurrences of clinical symptoms which may cause pain or indicate distress.
6. All procedures involving hazardous agents will be conducted in accordance with practices approved by the Safety, Health, and Environmental Management Office.
7. I certify that I am familiar with and will comply with all pertinent institutional, state and federal rules and policies.
8. The IACUC has oversight responsibilities for animal care and use, and may request consultation or feedback regarding the conduct of in vivo procedures, progress and accomplishments, and any problems encountered.

EPA Principal Investigator	Certification Signature Date
Exemption 6 Exemption 6	08/31/2015

Submitted: 09/01/2015

Certification:

Certification by EPA Supervisor (Branch Chief or Division Director) that the project described herein has been reviewed and approved on the basis of scientific merit:

Branch Chief/Division Director	*Approval Date*	*Phone Number*	*Division*	*Mail Drop*
Exemption 6	09/01/2015	Exemption 6	TAD	MD
	by Exemption 6 Exemption 6 Exemption 6 Exemption 6 Exemption 6 TP/USEPA/US	*Lotus Notes Address* Exemption 6 Exemption 6 Exemption 6 Exemption 6 Exemption 6 RTP/USEPA/US	*Branch* RTB	*Submitted to Branch Chief for Approval* 09/01/2015 10:50 AM

ATTACHMENTS

18-09-001 LAPR PI Response.pdf LAPRdesigns.pptx

Actions

First Update notification sent: 07/27/2016
Second Update notification sent: 08/31/2016
First 2nd Annual notification sent: 08/07/2017
Second 2nd Annual notification sent:

1st Expiration notification sent:
2nd Expiration notification sent:

History Log: